An electrochemical sensor for high sensitive determination of lysozyme based on the aptamer competition approach

1 Introduction

The molecular weight of lysozyme is about 14.4 kDa, containing 129 amino acid residues, and the isoelectric point is 11.0. It can destroy the cell wall of bacteria by catalyzing the hydrolysis of 1,4-β chains between n-acetyl-cell wall acid and N-acetylglucosamine residues in peptidoglycan and between N-acetylglucosamine residues in chitosan [1–4]. Lysozyme is widely found in cell secretions, such as saliva, tears, and other body fluids. Lysozyme is also widely used in pharmaceutical industry and food industry because of its antibacterial property. Lysozyme molecules are relatively small, and it is often used as a target detector in the study of aptamer sensors for protein detection [5–8].

The traditional methods of detecting lysozyme are gel electrophoresis, high-performance liquid chromatography, and immunoassay including immunoelectrophoresis and enzyme-linked immunosorbent assay [9,10]. However, although the sensitivity of immunoassay is high, it needs a long process of protein modification and is limited by the kinds of antibodies. Aptamer chain is easy to synthesize and modify and is usually more stable than antibody, so it is often used to detect lysozyme in recent years [9–17]. Zou et al. [15] combined the aptamer with 6-carboxyfluorescein to prepare an efficient lysozyme sensor. In addition, Wang et al. [16] obtained a fluorescent sensor using lysozyme aptamer. Li and his collaborators [17] fixed DNA complementary to lysozyme aptamer on the sensor; one end was modified with ferrocene lysozyme aptamer, and hybridized to the fixed chain. When there was a target, the aptamer chain fell off from the electrode surface, resulting in the weakening of electrical signal, and the detection limit of the sensor reached 0.1pM. However, it still takes more time to modify the electrical signal marker at one end of DNA [9,10].

From the end of the last century to the present, it has been found that nanomaterials have the characteristics that conventional macromaterials do not have in...
electromagnetic properties, catalysis, electricity, optics, and other aspects [16–20]. Therefore, nanomaterials are more and more widely used in catalysis, sensors, and other fields [21–25]. By controlling the synthesis conditions, many kinds of nanomaterials with different morphologies have been successfully prepared, such as nanoparticles with tooth shape, strip shape, multilateral shape, and empty shell shape. Because of the large specific surface area and high reactivity of nanoparticles, the amount of DNA modified on the electrode surface can be increased, thus improving the linear range and detection sensitivity of the sensor [26–28]. There is a strong covalent bond between gold particles and sulfhydryl group, and nanogold can be used as a probe to detect the target [29–34]. Carbon nanotubes have been widely used in the development of chemical biosensors because of their good biocompatibility, effective specific surface, and good conductivity [23,35–40].

Daunomycin (DNM) is a chemotherapeutic drug belonging to anthracycline antibiotics, which was first obtained from Streptomyces peucetius. DNM targets include DNA, membrane phospholipid, and protein. DNM has a planar molecular structure, which can be embedded between adjacent base pairs of complementary DNA (dsDNA), interferes with the process of DNA replication and transcription, and induces DNA strand breakage through oxygen-free radicals or top II media. In addition, DNM can interact with membrane phospholipids, thus affecting intracellular information pathways and can also react with a variety of proteins to induce apoptosis [41–53]. Based on the above characteristics, DNM is often used to treat some cancers.

In this study, the electrochemical sensor of lysozyme based on the mechanism of aptamer competition was prepared using the characteristics of normycin that can insert double-stranded DNA specifically and DNM as the electrical signal indicator [47–49]. The carboxylated multi-walled carbon nanotubes (MWCNTs) were modified on the glassy carbon electrode (GCE), and the complementary aptamer DNA with amino group was connected to MWCNTs. Because DNM is inserted into complementary DNA on the electrode, electrochemical signals are generated [50,51]. When there is a target, the aptamer combines with lysozyme with higher binding force, and the original complementary chain breaks down, resulting in the falling off of DNM inserted into the double chain, so the electrical signal is weakened.

2 Experimental

All the reagents were of analytical grade. Nitric acid, potassium ferricyanide, potassium ferrocyanide, concentrated sulfuric acid, ethanol, and N,N-dimethylformamide (DMF) were purchased from Macklin Co. Ltd. and used without purification (analytical grade). DNM is provided by Shanghai CDC. Lysozyme purchased from CETEC Biotechnology (USA) Co., Ltd. DNA and complementary DNA are synthesized by Biotechnology (Shanghai) Co., Ltd. The sequence of oligonucleotide used was 5′-HS-(CH₂)₈-ATCTACGAATTCTACGGGCCTAAAGAGTCAGAGTT ACTTAG-(CH₂)₈-NH₂-3′. The sequence of dsDNA was 5′-CTAAGTAACTCTGCAGAG-3′. MWCNTs were purchased from Shenzhen Nanotechnology Co., Ltd. 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and n-hydroxysuccinimide (NHS) were purchased from Sigma. Phosphate-buffer solution (PBS) was prepared by mixed stock solutions of 0.1 M disodium hydrogen phosphate and sodium dihydrogen phosphate.

Figure 1: Assembly process of lysozyme sensor.
The electrochemical experiment was carried out on the CHI 760C electrochemical analyzer (Shanghai Chenhua Instrument Co., Ltd., China). Three electrode system was adopted: modified electrode as working electrode, platinum wire electrode as counter electrode, and saturated calomel electrode as reference electrode (SCE); the solution used in the electrochemical impedance analysis was 0.1 M KCl containing 10 mM K$_4$[Fe(CN)$_6$] and 10 mM K$_3$[Fe(CN)$_6$]. Both cyclic voltammetry (CV) and differential pulse voltammetry (DPV) experiments use 0.1 M PBS as the test solution (containing 0.1 M KCl, pH 6.4).

The assembly process of electrode surface is shown in Figure 1. MWCNT (5 mg) was dispersed in 5 mL of anhydrous DMF for 1 h sonication; 5 μL of them was added to the GCE and placed in a drying oven for 24 h. After DMF volatilized, a thin layer of MWCNTs was formed on the GCE (denoted as MWCNTs/GCE). The aptamer DNA with a concentration of 20 μM was mixed with complementary DNA, heated to 94°C, and then cooled slowly at room temperature. Add 5 mL of mixed solution of 10 mM EDC and 10 mM NHS on MWCNTs/GCE to activate the carboxyl group on carbon nanotubes. After 10 min, take 5 μL of annealed complementary DNA and drop it on MWCNTs/GCE. Cover the electrode with plastic tube to prevent liquid volatilization and let it stand for 1 h. Then rinse with ultra-pure water. The electrode was recorded as dsDNA/MWCNTs/GCE. Finally, 5 μL of DNM solution with a concentration of 10 mM was dropped on dsDNA/MWCNTs/GCE, which was left for 5 min at 4°C, and the electrode was washed with ultra-pure water. DNM–dsDNA/MWCNTs/GCE was used to represent the modified electrode.

Ethical approval: The conducted research is not related to either human or animal use.

3 Results and discussion

EIS can reflect the process of electrode modification according to the change of electrode impedance. Figure 2 shows the impedance spectrum of the modified electrode in 10 mM [Fe(CN)$_6$]$_{4-}$/3$^-$ solution, with the frequency varying from 0.01 Hz to 10 kHz. It can be seen from Figure 2 that compared with the bare GCE, the conductivity of MWCNTs/GCE is significantly enhanced, and the semicircle of impedance is significantly smaller. When DNA was modified, the impedance reached 8,000 Ω, indicating that DNA was successfully modified on the electrode surface [51,52].

Figure 3 is a scanning electron micrograph of the surface of the modified electrode. Clean the surface of bare GCE. After the carbon nanotubes were modified, it was clear that the carbon nanotubes were uniformly distributed on the surface of the GCE. The length of
the carbon tube is about 1 µm, and the diameter is 50–100 nm. The morphology is beneficial to increase the specific surface area of the electrode.

The electrochemical behavior of the DNM–dsDNA/MWCNTs/GCE in PBS is shown in Figure 4a. DNM can produce two pairs of redox peaks. A pair of peaks in the range of 0.20–0.40 V is produced by the phenolic structure in DNM molecule. A pair of peaks between 0.60 and 0.70 V is related to the quinone structure. Figure 4b and c shows the DPV of the electrode. It can be seen from the figure that there are also two groups of peaks in DPV, which is consistent with the result of CV chart. The peak current of a pair of peaks of 0.60–0.70 V related to quinone structure is larger, and the peak current increases rapidly with the increase in DNM inserted into DNA double helix. Therefore, we choose DPV signal of the oxidation peak of this group of peaks for quantitative determination.

Add 20 µL lysozyme solution with different concentrations on DNM–dsDNA/MWCNTs/GCE, respectively, and wash the electrode with PBS solution after standing for 2 h at 25°C. Then put the electrode into PBS solution and test the DPV signal. The results are shown in Figure 5a. The response current of the electrode decreases with the increase in lysozyme concentration. Figure 5b shows the linear correction relationship between lysozyme concentration and response current. The detection range of lysozyme was 1–500 nM, and the detection limit was 0.5 nM. Under the same conditions, the lysozyme showed no electrochemical signal on GCE, MWCNTs/GCE, and dsDNA/MWCNTs/GCE. Table 1 shows the comparison of previous report with our result.

The effect of different scanning speeds on the oxidation peak current of DNM–dsDNA/MWCNTs/GCE in PBS solution (Figure 6) before and after lysozyme (Figure 7) detection was investigated. The oxidation peak current increases with the increase in sweep rate, while the peak potential is basically unchanged. The sweep speed is in the range of 100–600 mV/s, and the peak current has a good linear relationship with the sweep speed. This shows that the electrochemical reaction is controlled by adsorption [57]. In addition, when the sensor detects

![Figure 4](image1.png)

**Figure 4:** (a) CV of DNM–dsDNA/MWCNTs/GCE in PBS. Enlarged (b) negative scan and (c) positive scan of DNM–dsDNA/MWCNTs/GCE.

![Figure 5](image2.png)

**Figure 5:** (a) DPV curves of the DNM–dsDNA/MWCNTs/GCE toward to different concentrations of lysozyme. (b) Plots of concentrations of lysozyme with the peak current.

![Figure 6](image3.png)

![Figure 7](image4.png)
10 nM lysozyme, there is a good linear relationship between peak current and scanning speed in the range of scanning speed of 100–600 mV/s. It suggested the electrochemical reaction is controlled by adsorption as well.

The effective area of the electrode can reflect whether the layer by layer modification is successful. The effective area data of the electrode can be obtained by chronocoulometry in 0.1 M KCl + 10 mM K₃[Fe(CN)₆] solution. Figure 8 shows the timing Coulomb data of bare GCE and MWCNTs/GCE. The slope can be obtained by plotting the square of time (t) with current (I). The electrochemical effective surface areas of bare GCE and MWCNTs/GCE modified electrodes can be calculated as 0.00052

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<th>Method</th>
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<td>[52]</td>
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Table 1: Comparison of previous reports with the dsDNA/MWCNTs/GCE toward lysozyme determination

Figure 6: CVs of DNM–dsDNA/MWCNTs/GCE in the absence of the lysozyme with different scan rate (100, 150, 200, 250, 300, 350, 400, 450, 500, 550, and 600 mV/s).

Figure 7: CVs of DNM–dsDNA/MWCNTs/GCE in the presence of 10 nM lysozyme with different scan rate (50, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, and 600 mV/s).
and 0.00124 cm², respectively. The experimental results are in accordance with the expectation that the effective area of the electrode is increased and the sensitivity of the electrode is improved [41].

Nonspecific adsorption often interferes with the determination of target by biosensors. To verify the selectivity of the sensor to the target, 20 μL of 100 nM glucose oxidase (GOD), 100 nM immunoglobulin G (IgG), and 0.5 g/L bovine serum albumin (BSA) were successively added to DNM–dsDNA/MWCNTs/GCE under the same conditions. Allow to stand for 2 h. Then wash the electrode with PBS solution and measure the DPV signal (Figure 9). It can be seen from Figure 9 that the reduction in response current caused by higher concentration of GOD, 100 nM IgG, 0.5 g/L BSA is less than that caused by lower concentration of target. It is proved that the sensor has good selectivity and anti-interference.

The relative standard deviation of 5 nM lysozyme was 5.2%, which indicated that DNM–dsDNA/MWCNTs/GCE sensor had good reproducibility. When the sensor needs to be stored, it can be placed in a humid environment of 4°C. After 1 week of storage, the current response of the electrode is 95% of the current response value when the preparation is finished, which proves that the sensor has good stability.

4 Conclusion

The DNM–dsDNA/MWCNTs/GCE sensor developed in this work is a new type of unmarked lysozyme detector. The sensor uses DNM as the active substance of electrical signal, which has the characteristics of strong signal and high stability. The response range was 1–500 nM, the correlation coefficient was 0.9995, and the detection limit was 0.5 nM. Compared with the traditional electrochemical method, the sensor prepared in this chapter has a wide linear range, strong anti-interference, high sensitivity, and strong practicability.

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Conflict of interest: The authors declare no conflict of interest.

Data availability statement: The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

References


